THE EXPRESSION OF CD44s IN SQUAMOUS CELL CARCINOMA OF THE ORAL TONGUE AND ASSOCIATION WITH CLINICOPATHOLOGICAL FACTORS AND SURVIVAL OUTCOMES

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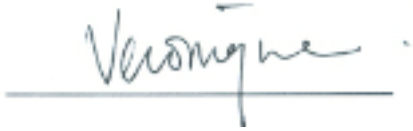
DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in university previously.



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SUMMARY

CD44 is a cell-surface molecule that functions as a receptor for hyaluronan- a major component of the extracellular matrix. Its interaction with hyaluronan and structural diversity confers it a wide range of functions. $CD44^+$ expression has been identified as a marker for a population of cells with cancer stem cell characteristics in head and neck squamous cell carcinoma (HNSCC). The aim of this study is to investigate the expression of CD44s in squamous cell carcinoma (SCC) of the oral tongue using immunohistochemistry, and to correlate CD44s expression with histopathological features and patient outcome.

Immunohistochemical analysis of CD44s expression was performed on tongue SCC tissue obtained from 51 consecutive patients who underwent surgical resection between Jan 2002 to Oct 2005. CD44s expression was based on staining intensity and percentage of tumour cells expressing CD44s. Expression of CD44s and its association with histopathological parameters were analysed using either the Chi-square test or Fisher's exact test as appropriate. The Kaplan Meier method was used to estimate survival distributions. Cox proportional hazard models were fitted to estimate hazard ratios to assess association of factors with each endpoint.

The median follow-up since surgery was 4.2 years. Intensity of CD44s staining and percentage staining varied among the samples. Intensity of staining was found to be a better indicator of outcome. Patients with strong

CD44s staining intensity had better overall survival compared to patients with low or moderate CD44s intensity (HR 0.32, Log rank p = 0.04). Strong CD44s intensity was also associated with better locoregional recurrence-free interval (HR 0.22, 95%CI 0.05 – 0.87; P = 0.029). There was no association between CD44s expression and adverse histopathological features.

We conclude that strong staining intensity of CD44s is an independent positive prognostic factor for overall and locoregional recurrence-free survival in oral tongue SCC.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
CD44s	standard CD 44
CSC	cancer stem cell
DNA	deoxyribonucleic acid
EMT	epithelial-mesenchymal transition
FACS	fluorescent-activated cell sorting
FFPE	formalin fixed, paraffin embedded
HA	hyaluronan
HIER	heat induced epitope retrieval
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
IHC	immunohistochemistry
IMRT	intensity-modulated radiation therapy
NOD/SCID	non-obese diabetic severe combined immunodeficient
SCC	squamous cell carcinoma

CHAPTER 1

INTRODUCTION

1.1 Head and Neck Cancers

Squamous cell carcinomas of the head and neck (HNSCC) are epithelial malignancies that arise from the paranasal sinuses, oral cavity, oropharynx and larynx. As a group they represent the 6th most common type of cancer in Western countries, accounting for an estimated 650 000 new cancer cases and 350 000 cancer deaths worldwide every year.[1] Oral cancer alone accounts for 270 000 new cases annually and 145 500 annually, the majority of which occur in developing countries.[1] These cancers are largely amendable to curative surgery or radiotherapy, with or without concurrent chemotherapy, when diagnosed at an early stage.

1.2 Squamous Cell Carcinoma of the Oral Tongue

1.2.1 Epidemiology and Etiology

Tongue cancer is the most common malignancy arising from the oral cavity in the head and neck. As with most cancers in the head and neck, squamous cell carcinoma (SCC) is the most common.[2] Worldwide, oral tongue SCC is an important cause of morbidity and mortality, although a significant geographical variation exists. Once considered to be a cancer on the decline in the developed world, a rising incidence suggests a continuing increase in the absolute numbers of cases to be treated in the coming decades.[3] In India, incidence rates among males of up to 6.5 per 100 000 per year have been reported. In parts of Europe, like France, the

male incidence rate is 8.0 per 100 000 per annum.[4] Based on the National Cancer Institute's (NCI) Surveillance Epidemiology and End Results (SEER) cancer statistics, within the United States, an estimated 12,770 new diagnoses of oral tongue SCC will made and an estimated 2050 men and women will die of oral tongue cancer in 2012.[5]

The etiology of tongue SCC is well described. Tobacco use, heavy alcohol consumption, poor nutrition, immunocompromised health states and viral infections have all been implicated in the carcinogenesis of squamous cell carcinoma of the tongue. Of these risk factors, tobacco and alcohol consumption are thought to account for more than 75% of oral tongue cancers.

1.2.2 Current opinions in management and therapy

The oral tongue is defined as the anterior two-thirds of the tongue that lies within the oral cavity. Tumours that arise from the posterior one-third, or base of tongue, are defined as oropharyngeal cancers. Patients with oral tongue SCC usually present earlier than those with tumours at the base of tongue, largely due to better visibility to both patient and clinician. Despite this, many patients still present at a late stage, perhaps because cancers in the early stage are frequently painless.

The treatment of tongue SCC is a multidisciplinary effort. The ability to

completely resect tumour with clear surgical margins is critical and directly affects survival.[6, 7] The choice of adjuvant therapy – radiation or concurrent chemo-irradiation depends on the operative findings and histology. In the last decade, significant improvement in every field has been made. Advances in surgical reconstruction with microvascular free-tissue transfer options allow for large tumours to be resected with adequate margins. Randomised trials have also demonstrated that the concurrent use of cisplatin with post-operation radiation therapy benefits patients with poor prognostic features.[6, 8, 9] Patients now receive this if adverse histological features are present. The delivery of radiation in the form of intensity-modulated radiation therapy (IMRT) has also effectively allowed for high dose radiation to affected area with reduced radiation dose to adjacent sensitive structures such as the salivary glands, aerodigestive mucosa and spinal cord.[10, 11] Despite these advances, SCC of the oral tongue remains a disease with significant locoregional recurrence rates and poor survival.

Local and/or regional disease recurs in 50-60% of patients with advanced disease treated with combined modality therapy.[6, 8] Locoregional recurrences result in significant morbidity and mortality as speech and swallowing are frequently affected. A percentage of such patients may be treated with re-resection. Re-irradiation with or without cisplatin is also being explored.

Apart from improvements in the therapeutic fronts, research is being done to explore why oral tongue SCC recurs so frequently despite aggressive

multimodality therapy, and there is ongoing efforts to seek new molecular therapeutic targets. The cancer stem cell theory is a recent development in cancer biology that may redefine our understanding and approach to treating tongue cancer.

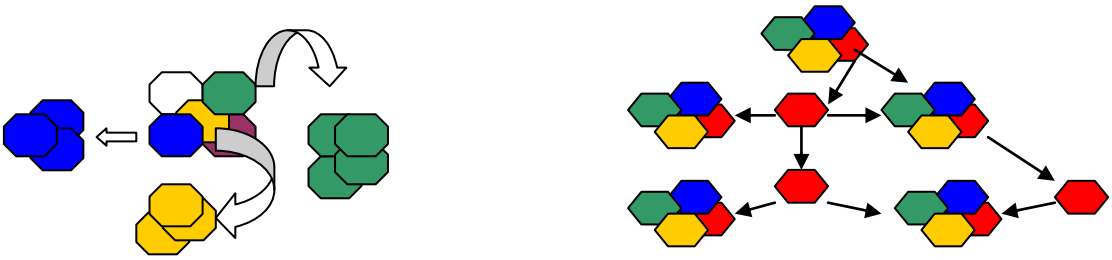
1.3 Cancer Stem Cells

Stem cells have the ability to generate large numbers of mature cells through a hierarchy of proliferation and differentiation, while retaining the ability to self-renew in order to maintain the stem cell pool. Such hierarches of development exist both in the embryonic and adult states – as pleuripotent cells in the embryo developing into specialized cells, and as a repair system for the body to replenish adult tissues.

The cancer stem cell hypothesis suggests that a tumour may be viewed as an aberrant organ that is sustained, in a way similar to normal tissues, by a subset of biologically distinct stem cells (cancer stem cells). These cells constitute a small subset of the tumour and drive tumourigenesis, producing both stem cell progenies and differentiated, non-tumourigenic progenies that make up the bulk of the tumour (Figure 1).[12] This model of tumourigenesis challenges the traditional model of carcinogenesis where adult somatic cells are thought to acquire the ability to self renew and generate a tumour due to the accumulation of multiple mutations. Malignant transformation of multiple clones were thought to account for the

phenotypic diversity within a tumour, and each cell regarded as having the ability to proliferate and metastasize. Should the stem cell theory be true, a paradigm change in how we view and treat cancer is in order. The success of therapies would no longer be determined by mere shrinkage of tumours or metastatic deposits, and not all cells within a tumour should be considered equal. Rather, it is this precise subset of cancer stem cells that we would need to characterize an target for elimination (Figure 2).

Figure 1 Two models of heterogeneity in solid tumours

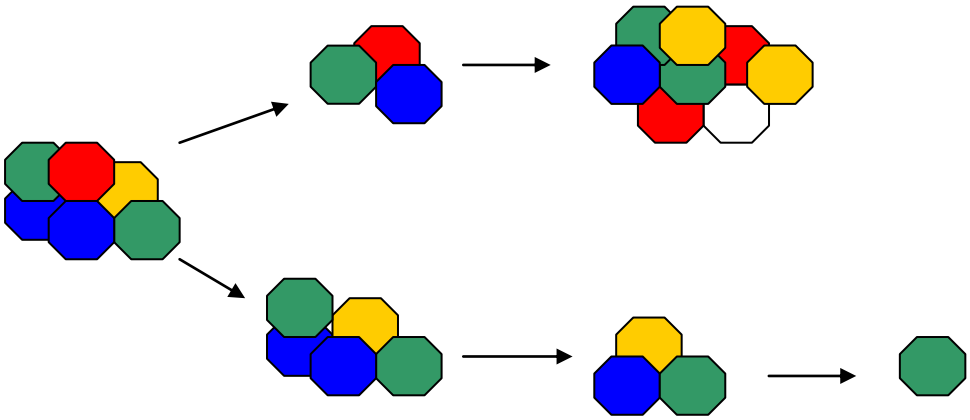


a) Stochastic model b) Cancer stem cell model

In the traditional model of tumourigenesis (a), each cancer cell has the potential to mutate, proliferate and form a new clone. The cancer stem cell model (b) hypothesizes that only the cancer stem cell (red) is able to drive tumourigenesis, producing both stem cell progenies and heterogenous non-tumourigenic progenies that constitute the bulk of the tumour.

Figure 2. Cancer stem cells and therapy

A) Therapy does not effectively kill cancer stem cell (red). Tumour regenerates despite initial shrinkage.



B) Therapy that targets cancer stem cells (red) may not shrink significantly initially, but the tumour fails to repopulate and eventually degenerates.

1.3.1 Evidence for cancer stem cells

The first documentation that only a small subset of cancer cells is capable of extensive proliferation leading to tumour formation came from studies of acute myeloid leukemia and multiple myeloma. Evidence of leukemic stem cells was elegantly demonstrated by Bonnet and colleagues when they isolated $CD34^{+}CD38^{-}$ cells from leukemic patient samples and showed that *only* these cells, which constitute a variable proportion of acute myeloid leukemic cells – 0.2% in one patient, were able to transfer acute myeloid leukemia from patients to Non-obese diabetic severe combined

immunodeficient (NOD/SCID) mice.[13] This demonstrated conclusively that not all cells had similar clonogenic capabilities, and that a specific, identifiable group of cells had enriched capacity to form clones while the majority of leukemic cells lack the ability to proliferate and transfer disease.

Similar observations were made of some solid cancers. In breast cancer, similar to leukemia and other hematological malignancies, tumourigenic and non-tumourigenic populations of breast cancer cells were isolated based on their expression of cell surface markers. In many cases of breast cancer, only a small subpopulation of cells, characterized by $CD44^{+}CD24^{-}$ had the ability to form new tumours.[14, 15] Furthermore, when these tumourigenic cells were injected into NOD-SCID mice, the tumours formed contained multiple cells lines. This work strongly supported the existence of cancer stem cells in breast cancer.[14] Further evidence for the existence of cancer stem cells occurring in solid tumours has been demonstrated in malignancies of the central nervous system (CNS). Using culture techniques similar to those used to culture normal neuronal stem cells, it was shown that neuronal CNS malignancies contain a small population of cancer cells, identified by $CD133^{+}$ surface expression, that are clonogenic in vitro and initiate tumours in vivo. $CD133^{-}$ cells did not exhibit these properties. [16]

The defining characteristics of this group of cells with stem-like qualities are their ability to give rise to a bulk mass of differentiated tumour cells,

self-renew on serial transplantation studies, and be able to do so in small numbers whereas tumour cells not of this fraction lack this capacity despite greater numbers being transplanted. These stem-like cells may be identified by specific markers. Fluorescent-activated cell sorting (FACS) using specific antibodies directed against cell surface antigens such as CD34, CD133 and/or CD44 is a commonly employed technique to sort and isolate the small population of cells with stem cell-like properties.[15-19] The Hoechst dye efflux technique is another method used to isolate the side population (SP) cells that are enriched in tumour-initiating capability compared to non-SP cells, although it is likely these do not represent a pure population of so-called cancer stem cells. Tumours in which such side populations of tumourigenic cells have been identified and characterized include the CNS, breast, ovary, prostate, pancreas, colorectal and head and neck cancers.[15-18, 20, 21]

1.3.2 Pathobiology of cancer stem cells

Having characterized in tumours a sub-population of cells with stem cell-like properties and christening them as cancer stem cells (CSCs), research has sought to better understand this group of highly tumourigenic cells. Their origin is itself debated – whether they arise through mutations of normal somatic stem cells, or did the malignant stem cell-like characteristics develop from an accumulation of genetic mutations and de-differentiation of mature cells.

Work done by Vogelstein and Weinberg estimated that three to six genetic mutations are required to induce malignant change to a normal human cell.[22, 23] In order to become malignant, cells need to exist long enough to accumulate these genetic changes – an argument that supports normal somatic stem cells as the cells of origin as the hierachical stem cell concept indicates that stems cells are the only cells that remain and survive long enough to accumulate these mutations. In addition, signaling pathways found to be critical for embryonic development and stem cell / progenital cell renewal, such as the Notch, Sonic hedgehog (Shh) and Wnt signaling pathways are also associated with oncogenesis, suggesting that the cells of origin of a cancer stem cell likely already had such self-renewing capability, thus providing further indirect evidence that cancer stem cells arise from normal somatic stem cells.[24, 25] Conversely, other studies suggest that cancer stem cells may originate through de-differentiation of mature cells. Keratinocytes that are downstream from their progenitor stem cells have been shown both in vivo and in vitro to acquire oncogenic events that induce stem cell-like renewal capacity.[26, 27]

Apart from initiating tumourigenesis, cancer stem cells have also been linked with the metastatic dissemination of epithelial cancer cells. The epithelial-mesenchymal transition (EMT) is the process where a polarized epithelial cell assumes a mesenchymal cell phenotype. It has a crucial role in embryogenesis - in the differentiation of various tissues and organs, and in tissue repair.[28] During EMT, cell-cell and cell-extracellular matrix

contacts are broken, and epithelial cells migrate to other locations in the body.[29] Recent studies have suggested that the activation of signaling pathways that induce EMT generates stem cell-like properties in non-tumourigenic mammary epithelial cells.[30, 31] In immortalised human mammary epithelial cells, the activation of EMT resulted in the acquisition of the $CD44^{+}/CD24^{-}$ stem cell phenotype. In essence, EMT endows cells with migratory and invasive properties, induces stem cell properties, and prevents apoptosis and senescence. It is implicated in cancer where the mesenchymal state is associated with the capacity of cells to migrate to distant organs and maintain stem cell qualities, allowing their subsequent differentiation into multiple cell types during development and the initiation of metastasis.

Besides attempts to characterize the role of cancer stem cells in the initiation, progression, invasion and metastasis of cancer, there is also much interest in the effect of current therapies upon these cells. If by analogy to normal stem cells, cancer stem cells should be inherently resistant to chemotherapy and radiation therapy through mechanisms that serve to protect stem cells from DNA and cellular damage, they would conceivably be resistant to the traditional oncologic treatment strategies of radiotherapy and chemotherapy. Tumour recurrences may then be attributed to surviving cancer stem cells that have escaped multimodal therapy.

Phillips et al. provide evidence that CSCs may be intrinsically radioresistant.[32] In their study, nonadherent cells isolated from two established breast cancer cell lines and propagated as mammospheres, having a higher fraction of $CD24^{-/low}/CD44^{+}$ cells (previously identified as cancer-iniatiating cells) were compared with adherent cultures. Both cell populations were irradiated as single-cell suspensions, removing the complicating factor of low oxygen tension at the centre of spheroids. When irradiated in vitro, the cells arising from spheroids were more radioresistant. Furthermore, fractionated radiation appeared to increase the percentage of nonadherent $CD24^{-/low}/CD44^{+}$ cells in monolayer cell cultures, suggesting that the relative radioresistance of this population of cells may lead to their expansion after a course of radiotherapy.[32] In another study, Bao et al. studied xenografts from primary glioblastoma multiforme specimens. They found that $CD133^{+}$ cells (previously established to be the tumourigenic population in primary glioblastoma multiforme) were radioresistant compared with $CD133^{-}$ cells.[33] Importantly, they showed that $CD133^{+}$ cells accumulated after irradiation both in vitro and in vivo. In addition, they demonstrated that the modest enrichment of $CD133^{+}$ cells after irradiation has biological relevance by showing that a slight increase in the percentage of $CD133^{+}$ cells in suspensions used to initiate tumours dramatically increased their growth rate. The relative radioresistance was further explored by investigating molecular markers of radiation damage. It was concluded that $CD133^{+}$ cells could activate DNA damage checkpoint responses to a greater degree that $CD133^{-}$ cells and thus repair DNA damage more efficiently.[33]

Stem cells from various normal tissues also tend to be more resistant to chemotherapeutics than their mature non-stem counterparts. The purported reasons include a higher level of antiapoptotic proteins and the presence of multidrug resistance (MDR) transporters that reduce the plasma membrane permeability to cytotoxic compounds.[34, 35] The protein glutathione-S-tranferase has also been implicated in intrinsic chemoresistance.[36] To date, the exact mechanisms of chemoresistance in tumour initiating cells have yet to be entirely elucidated, and extrinsic factors of the microenvironment may conceivably contribute.[36, 37] A better understanding of the complex interaction of cancer stem cells and chemotherapeutics is therefore essential to avoid tumour relapses driven by cancer stem cells having escaped multimodality therapy.

1.3.3 Cancer stem cells in Head and Neck Squamous Cell Carcinoma

In head and neck squamous cell carcinoma (HNSCC), Prince et al first isolated a highly tumourigenic subpopulation of cancer cells that had cancer stem cell properties.[20] Using the cell surface antigen CD44 that was previously demonstrated to be a useful cell surface marker for breast cancer stem cells (CSC), single-cell suspensions from HNSCC specimens were stained with an antibody to CD44. Flow cytometry analysis showed that HNSCC cells were heterogeneous for CD44 expression, with tumours comprised typically of < 10% CD44+ cells. The tumour cells were sorted to

purity using fluorescence-activated cell sorting (FACS), and cells with CD44 surface antigens and those without were injected into NOD/SCID mice. The $CD44^+$ group of cells were highly tumourigenic while the $CD44^-$ cells were not. In addition, the $CD44^+$ cells formed tumours with a mixture of $CD44^+$ and $CD44^-$ cells, and serial retransplantation of both populations indicated that only the $CD44^+$ population could initiate new tumours in vivo.[20] This experiment demonstrated that a specific subpopulation HNSCC cells, identifiable by their cell surface antigen CD44, had tumourigenic potential whereas cells not expressing the cell surface antigen were non-tumourigenic. That is, phenotypically distinct tumourigenic populations exist within head and neck squamous cell tumours. Serial transplantation of the purified subpopulation of $CD44^+$ cells generated new tumours, indicating that it is a self-renewing population.[20] Although these $CD44^+$ cells possess properties classically attributed to stem cells, the relatively large number of cells (>5000) needed to generate a new tumour in immunodeficient mice suggests that perhaps only a fraction of cells within population may be true CSCs, and that further refinement is likely required to precisely isolate HNSCC cancer stem cells. Aldehyde dehydrogenase (ALDH), a cytosolic enzyme, has also shown to be a useful marker to identify cancer cells with stem-like qualities in head and neck cancer.[38] $ALDH^+$ cells from patients with HNSCC showed enhanced tumourigenesis and radioresistance when compared to ALDH- cells.[38, 39] Furthermore, the knockdown of Snail decreased expression of ALDH and has been shown to inhibit cancer stem cell-like properties and the tumourigenicity of $CD44^+ALDH^+$ cells. [39]

The presence of an identifiable, phenotypically distinct subpopulation of tumourigenic cancer stem cells in head and neck cancer has clinical implications. It suggests that the presence of small numbers of these cells under the right conditions may lead to tumour repopulation and relapse whereas large numbers of non-tumourigenic cells may not necessarily be harmful. In the treatment of these tumours, attention should then be paid to the effective elimination of these cancer stem cells. If indeed they are more resistant to radiotherapy and chemotherapy, new treatment strategies to target and sensitise these cells may necessary. It would also mean a change in the measurement of success of treatment – from tumour shrinkage to a measurement of the elimination of cancer stem cells. New ways to identify this population of cells in situ in patients are thus needed.

The cell surface markers are also important. They may potentially be a prognostic marker, or aid in treatment selection. The use of these surface markers as a target for therapeutic agents has also been explored.[40] In essence, a better understanding of the intrinsic and extrinsic mechanisms of how these tumourigenic cells resist conventional treatments, self renew and metastasize may allow for the development of novel therapies and translate to less relapses and improved survival for patients with this devastating disease.

1.4 CD44

1.4.1 The transmembrane protein: CD44

CD44 is a type I transmembrane protein. It is a ubiquitous, abundant and functionally important cell surface receptor. A product of a single gene at 11p13, CD44 exists as several isoforms. It is encoded by at least 20 exons, with the first and last 5 being invariably expressed; their product is referred to as the “standard” or “haematopietic” form of CD44 (CD44s or CD44H correspondingly). Alternative splicing of the remaining intervening 10 exons give rise to a variety of CD44 isoforms that are named variants 1 to 10 (v1- v10). The structural diversity of CD44 is further amplified by posttranslational glycosylation and glycoaminoglycan attachment.[41, 42]

CD44 is a molecule of considerable interest as its structural diversity confers it a great functional spectrum. As a transmembrane protein, it has ligand binding capacity and plays a role in signal transduction pathways. It has been shown to mediate homotypic cell-cell adhesion, trigger heterophilic adhesion events (eg. between endothelial cells and leucocytes), act as a signaling molecule through tyrosine kinases, interact with EGFR leading to activation of EGFR-dependent signaling cascades, induce cytokine expression, and also induce cell proliferation and motility.[43-45] CD44 has been shown to be expressed in a wide variety of tissues and has been linked with various human diseases and

malignancies. In recent years it has also been implicated as a cancer stem cell marker.[14, 20]

CD44 acts mainly as a receptor for hyaluronan – a major component of the extracellular matrix.[46] Hyaluronan is enriched in many types of tumours, and in cancer patients, hyaluronan concentrations are usually higher in malignant tumours than in corresponding benign tissue.[47] The size of the hyaluronan fragment to which CD44 binds provides a physiologically important switch between its adhesive and signaling functions.[48, 49] Binding to hyaluronan polymers usually leads to cell adhesion, while binding to low molecular weight hyaluronan, a result of tissue damage and consequent degradation of the extracellular matrix, leads to CD44 signaling and activation of the immune system.[46, 48, 49] The binding of hyaluronan results in conformational changes or a redistribution of CD44 in the cell membrane and influences CD44-mediated signal transduction.

The cytoplasmic partner molecules of CD44 are the cytoskeleton proteins ankyrin and ezrin, radixin and moesin (ERM proteins). These proteins regulate cell shape, adhesion, migration and motility. Upon binding with hyaluronan, reorganisation of the cytoskeleton is initiated, and CD44 is guided to the leading edge of the migrating cells. Experimental work in vitro has demonstrated that specific isoforms of CD44 renders metastatic potential to non-metastasizing cell lines.[50] The role of CD44 in neoplastic metastasis was further supported when non-cytotoxic antibodies specific for the CD44 variant inhibited formation of secondary foci when injected

with tumour cells.[51] Biologically, specific hyaluronan-mediated CD44 activation of signaling events have been implicated in cellular adhesion, growth, survival, invasion and migration – all obvious prerequisites for metastasis.

1.4.2 CD44 as a cancer stem cell marker

The evidence that CD44 is expressed on cancer stem cells is recent, and raises the question of its role - as a mere cell surface marker, or having a functioning role in conferring stem-like qualities. It has been credited for maintaining and modulating the microenvironment of cancer stem cells, playing a role in antiapoptosis, conferring chemoresistance and being crucial to epithelial-mesenchymal transition.

The microenvironment of normal stem cells, or “niche”, maintains their quiescent and undifferentiated state while conferring proliferation and differentiation potential. A similar microenvironment exists for cancer stem cells. These niches are rich in hyaluronan, and hyaluronan-CD44 association facilitates maintainance of the necessary tumour matrix.[52] As a transmembrane proteoglycan, CD44 allows the local concentration of glycosaminoglycan-associating proteins, increasing the capacity of these ligands to interact with their receptors, thereby lowing the threshold for signal transduction. The binding of such ligands, including osteopontin and

vascular endothelial growth factor, are of interest in the metastatic process.[53-55]

Apart from maintaining the niche matrix surrounding cancer stem cells, CD44 is also involved in critical signal transduction pathways that confer stem-like characteristics. There is evidence that hylauronan-CD44 binding triggers pathways that lead to the transcription of the oncogenic microRNA miR-21 and a tumor suppressor protein (e.g. PDCD4: program cell death 4) reduction.[56] These events initiate the up-regulation of the inhibitor of apoptosis (IAP) proteins and mutidrug-resistant protein 1 (MDR1), resulting in anti-apoptosis and resistance to chemotherapeutics.[56, 57]

The abberant activation of epithelial-mesenchymal transition (EMT) facilitates metastasis by breakdown of cell-cell and cell-extracellular matrix contacts and confer cells the capacity to invade and ultimately metastasize to distant sites.[29, 58] CD44 and hyaluronan are important in regulating EMT.[52] In breast cancer cells the EMT phenotype is associated with a strong CD44 upregulation, and may be inhibited by CD44-specific antibodies.[59, 60] The inhibition of hyaluronan synthesis also reduces EMT and metastasis formation.[61, 62] These findings provide evidence for a role for CD44 and hyaluronan in EMT.

CD44 and its isoforms have been studied in various cancers. It has also been studied by several groups for its role in head and neck SCCs and in the various subsites of HNSCC. Previous studies exploring the clinical

significance of CD44 expression in head and neck tumours have shown conflicting results. Some reported poorer outcomes with low expression of CD44.[63-67] Others demonstrated a decreased survival and increased nodal metastasis with high CD44 expression.[68, 69] With its newly elucidated role as cancer stem cell marker in head and neck cancer, it may potentially have important clinical implications as a prognostic marker and therapeutic target. Yet other studies have described its ubiquitous expression on both benign and malignant head and neck epithelial as evidence that its value as a cancer stem cell marker be reconsidered.[70]

1.5 HYPOTHESIS

CD44 is a molecule with a spectrum of functional effects. It has been used as a marker for the identification of a subpopulation of cells with tumourigenic properties in head and neck cancer, and has been referred to as a cancer stem cell marker.

The immunohistochemical expression of CD44 may be indicative of the proportion of cancer stem cells within the tumour. It may therefore have prognostic significance in the clinical outcomes of patients with oral tongue SCC, be a potential biomarker for increased resistance to chemotherapy and radiotherapy, and may be associated with adverse histopathological features.

AIM

This study aims to confirm that specimens of oral tongue SCC have variable immunohistochemical staining of CD44s. It aims to determine if CD44s expression is associated with known adverse histopathologic features, and if it correlates with the clinical outcomes of survival and tumour recurrence and may thus be a prognostic marker.

It also aims to determine if CD44s expression in head and neck SCC is associated with clinical response to chemotherapy or radiotherapy.

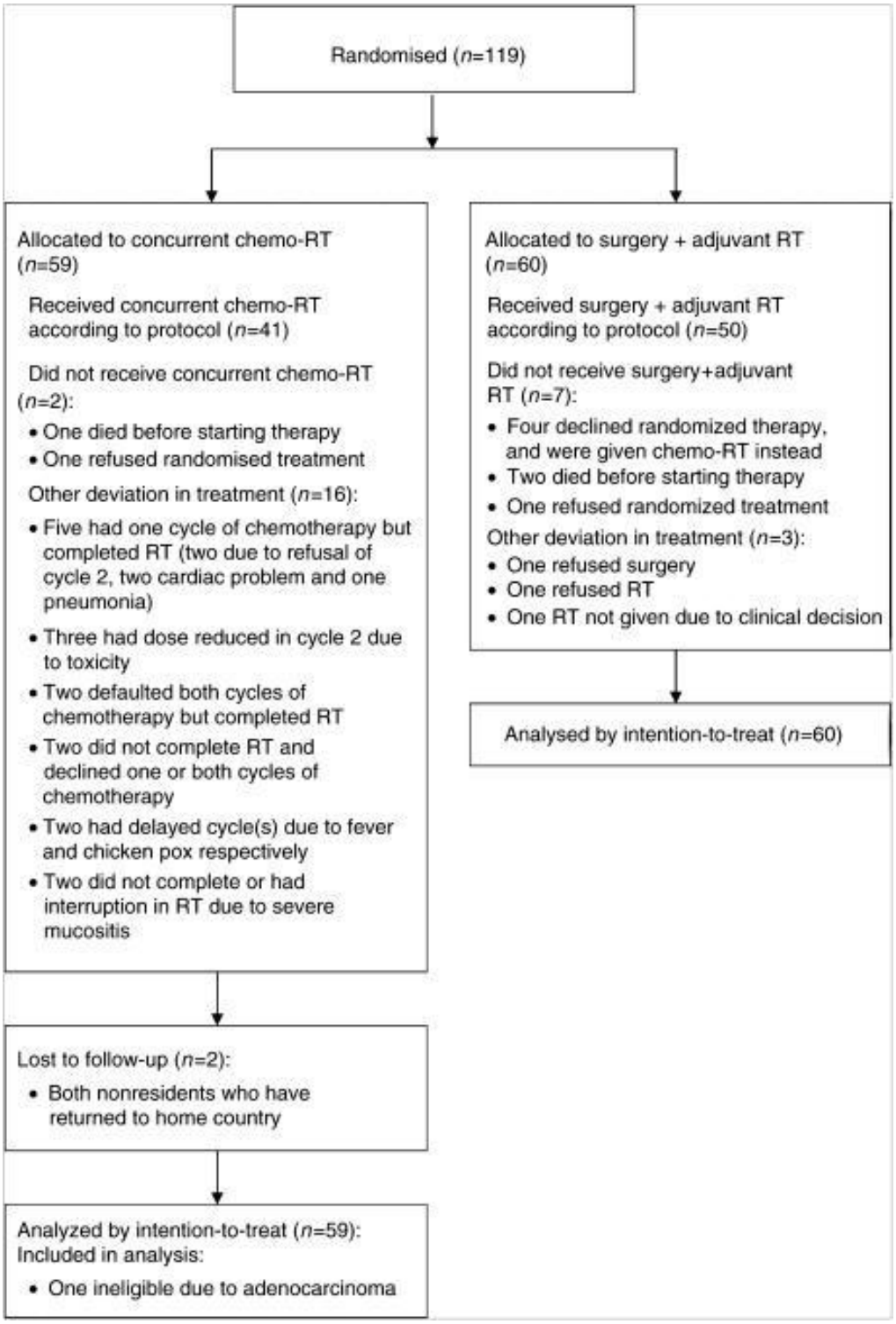
CHAPTER 2

MATERIALS AND METHODS

2.1 Initial study design

In order to evaluate if CD44s expression in head and neck SCC correlated with clinical response to chemotherapy or radiotherapy, the initial dataset and experiments were based on archival tissue of patients who had been enrolled in a multicentre prospective randomized trial which investigated the use of surgery and adjuvant radiotherapy vs upfront concurrent chemoradiotherapy for locally advanced, resectable head and neck SCC.[71] Patients eligible for the trial had newly diagnosed, histologically proven squamous cell carcinoma of the head and neck that were locally or regionally advanced, not metastatic, and were deemed resectable. Tumor "resectability", while largely subjective, was determined after clinical and radiological evaluation at a multidisciplinary tumor board with input from a panel of surgical oncologists, radiologists, medical oncologists and radiation oncologists. Tumors from all head and neck subsites except the nasopharynx and salivary glands were included.

Patients who met the above criteria had been randomized into the two treatment arms: the standard (S) arm consisting of radical surgery with adjuvant radiotherapy, and the experimental arm (C) of combination chemotherapy (cisplatin and 5-FU) administered concurrently with radical radiotherapy.



Soo KC BJC 2005 [71]

Figure 3. Trial profile of SHN01, a multicentre prospective randomized trial which investigated the use of surgery and adjuvant radiotherapy vs upfront concurrent chemoradiotherapy for locally advanced, resectable head and neck SCC

The (S) arm received surgery consisting of wide resection of the tumor with frozen section control during the surgical procedure to ensure clear margins. Comprehensive neck dissection with removal of levels I to V lymph nodes was performed unilaterally or bilaterally as indicated. Adjuvant radiotherapy was given to the primary tumor site and upper neck at 2 Gy per fraction, 5 days a week to a total of 60 Gy in 30 fractions over 6 weeks. Radiation therapy commenced as soon as adequate healing had been established, and not more than 6 weeks after surgery. Fields were reduced to exclude the spinal cord at 40 Gy and a posterior electron-matching field was applied. The dose to clinically uninvolved nodal region was 50 Gy. In patients with disease extending low down the neck, an anterior based AP/PA field was treated to a dose of 50 Gy, followed by lateral fields in another 10 Gy that did not include the spinal cord in the treatment volume. Patients who had positive surgical margins had the dose to the area at risk escalated to 70 Gy using reduced volumes. The lower anterior neck was treated if there was nodal disease present in the upper neck at 2 Gy per fraction to a total of 50 Gy in 25 fractions over 5 weeks.

Patients randomized to the (C) arm received radiotherapy similar to the (S) arm except that the total dose to the primary tumor and upper neck was 66 Gy in 33 fractions over six and a half weeks, with involved nodes receiving at least 60 Gy of radiotherapy. Two cycles of chemotherapy consisting of cisplatin 20mg/m^2/day and 5-fluorouracil 1000mg/m^2/day were given as a

continuous intravenous infusion over 96h on days 1 and 28 of radiotherapy.

Patients who received concurrent chemoradiotherapy underwent examination under anesthesia 6-8 weeks post treatment to evaluate tumor response. A complete response is achieved if there was complete disappearance of all clinically detectable tumour. Patients with persistent disease at the primary site were offered salvage surgery. All patients who had nodal disease at the onset would undergo elective neck dissections regardless of response to concurrent chemoradiotherapy.

Upon completion of primary treatment, the patients were followed monthly for the first year, two monthly for the second year, three monthly for the third and six monthly thereafter by all members of the multidisciplinary treatment team. Clinical examinations were performed at each visit with radiological investigations done when indicated. Suspected recurrences were biopsied and patients who developed locoregional disease subsequently were considered for appropriate salvage surgery.

Between 19 August 1996 to 21 February 2002, 119 patients were recruited to this clinical trial. Sixty patients were randomized to undergo surgery with adjuvant radiotherapy (S). Fifty-nine patients were assigned to primary treatment by concurrent chemoradiation (C). A review of these patients' pathological and clinical data including follow-up information was obtained from the National Cancer Centre Singapore head and neck cancer

database and electronic medical records. By July 2008, the median follow up time for the (S) arm was 7.1 years (range: 0.1 to 11.4 years) and that for the (C) arm, 8.7 years (range: 0.2 to 11.3 years).

Archived hematoxylin and eosine (H&E) histopathologic sections of tumour tissue from the trial patients were retrieved. Histological sections of the surgical specimens were examined by a senior consultant pathologist (HJS) and the most representative section of tumour was identified. For patients who had been randomized to upfront concurrent chemoradiotherapy, only the biopsy specimen of the tumour were available. The appropriate formalin-fixed, paraffin-embedded (FFPE) tissue blocks were then retrieved.

Microtome sectioning of the FFPE specimens were performed and 4um-thick sections were mounted on Super Frost/Plus-slides (Menzel, Braunschweig, Germany). To facilliate adhesion, the slides were dried by incubating for 12 h at 37°C then at 60°C for an hour. Sections were deparaffinised in xylene and rehydrated in a decreasing alcohol series.

Immunohistochemical validation was performed on a range of normal tonsillar tissue and achival head and neck SCC tumours. Unfortunately, attempts to optimize the immunohistochemical protocol for CD44s staining on the archival tissue failed. The main problem encountered was a consistent lack of staining on all the archived tissue. We employed heat-induced epitope retrieval (HIER) techniques using microwave heating,

vegetable steamers and water baths with varying pH buffers, epitope retrieval solutions, time and temperature combinations. Good staining on the positive controls (tonsillar tissue from 2008) were consistently obtained despite corresponding lack of staining of the all the archival tissue. Such disconcordance suggested that the problem was not from ineffective antigen retrieval, inactive antibodies or errors in the IHC protocol. It was likely that unalterable conditions of the archived tissue, such as inadequate fixation techniques, or epitope alterations during embedding with resultant failure to restore immunoreactivity accounted for the lack of staining. The fact that minimal tissue was available for the patients who underwent primary concurrent chemoradiation therapy (patients only had diagnostic biopsies) further compounded the difficulties in optimizing a suitable protocol. A decision was then made to use a different set of patients to investigate the correlation of CD44s expression with histopathological features and survival outcomes.

In the formulation of a new experimental plan, the following factors were considered. Firstly, tissue from one subsite within the head and neck was desired. This would minimize the confounding effect of inherent heterogeneity between the different subsites. Secondly, adequate tissue samples of the primary tumour should be available. Thirdly, specimens from the recent past should be used to minimize significant differences in fixation and embedding techniques and loss of epitopes with prolonged (possibly suboptimal) storage.

As such, we decided to investigate the expression of CD44s expression in oral tongue SCC. By restricting tumours to one subsite of the head and neck, the biases that may influence survival outcomes are minimized. Also, oral tongue SCC is the most common head and neck cancer, and any significant finding potentially has greatest clinical impact. Furthermore, the mainstay of treatment of oral tongue SCC to date is primary surgical resection, and adequate tumour tissue from an enbloc resection would be available.

To avoid similar problems with epitope retrieval, hypothesized to be due to poor fixation techniques in the 1990s and suboptimal, prolonged storage, we retrieved a random sample of 10 oral tongue specimens from the recent years of 2002 and 2003. These years were also selected as patients from then would provide adequate follow up data for meaningful survival analyses. We did a trial of IHC staining for CD44s, and a protocol was successfully optimized, with appropriate positive and negative controls.

2.2 Patients and specimens

The current study is based on formalin fixed, paraffin embedded (FFPE) histopathological specimens from consecutive patients with oral tongue squamous cell carcinoma treated with radical excision of the primary tongue lesion at the Department of Surgical Oncology, National Cancer Centre Singapore and Department of General Surgery, Singapore General Hospital, within the period of January 2002 – December 2005. A retrospective review of these patients' pathological and clinical data including follow-up information was obtained from our Head and Neck Cancer Database and electronic medical records.

For the purpose for this study, we included all patients with histologically proven SCC oral tongue treated with surgical resection upfront. Patients with non-SCC tongue cancers, non-tongue SCC cancers of the head and neck, those with incisional biopsies only, or had prior treatment with chemotherapy and/or radiation therapy were excluded from this study.

2.3 Immunohistochemistry

The hematoxylin and eosine (H& E) stained histopathologic sections of the surgical specimens were examined by a senior consultant (HJS) at the Department of Pathology, Singapore General Hospital. A representative section of the primary tongue SCC was identified and the corresponding FFPE block retrieved. For immunohistochemistry, 4-um thick sections

were mounted on Super Frost/Plus-slides (Menzel, Braunschweig, Germany) and incubated at 60°C for an hour then at 37^{o}C for 12 hours. The histological sections were deparaffinised in xylene and rehydrated in a decreasing alcohol series. The sections were then submitted to antigen retrieval by steaming for 20 mins in a commercial Epitope Retreival Solution (Tris/EDTA-based buffer pH9 Novocastra™, Wetzlar, Germany) and cooled for 20 mins. The samples were then washed with phosphate-buffered saline (PBS, pH 7.2) twice for 3 minutes on a belly dancer. Each histologic section upon the slides was then outlined with a delimiting pen (Dako™, Glostrup, Denmark). Endogenous peroxidases were blocked by 3% peroxidase treatment for 15 min. The samples were again washed in PBS for 5 min then incubated in 1.5% horse serum for 30 min to prevent non-specific antibody binding. A monoclonal mouse antibody with specificity to CD44s (Novocastra™ NCL-CD44-2, Wetzlar, Germany) diluted to 1:100 with Tris-buffered saline (IHC Diluent, Novocastra RE7133, Wetzlar, Germany) was used as the primary antibody. The slides were incubated for 30 min at room temperature then washed with PBS and further incubated with Dako REAL™EnVision™ Detection System for 30 min (Dako, Glostrup, Denmark). Immunostaining was visualized with 3,3-diaminobenzidine for 5 min and the reaction was stopped by washing in running water. Counterstaining was performed with hematoxylin, dehydrated and mounted with DePex (BDH Ltd, Poole, UK). In each staining series, a known CD44s positive tongue SCC specimen served as a positive control and the same tongue SCC section processed as per

protocol but without primary antibody incubation served as a negative control.

All tumours were re-evaluated for histological type, grade and immunohistochemical staining by a senior consultant histopathologist blinded to the clinical data. The immunoreactivity of the tumour cells for CD44s was scored according to the percentage of CD44s staining (score ranging from 0% to 100%), and the intensity of all cells that had a positive staining was estimated on a semi-quantitative three-grade scale from 1 to 3+ (1: weak staining, 2: moderate staining, 3: strong staining).

2.4 Statistical analysis

Comparisons of CD44s intensity between patients in different histopathological characteristic groups were performed using either the Chi-square test or Fisher's exact test as appropriate.

Mann-Whitney U test was used to detect any significant differences in the CD44s percentage between patients with and without the presence of angioinvasion, perineural invasion or nodal disease.

Kruskal-Wallis test was used to compare CD44s percentage between patients in the 3 tumour grade groups (well-differentiated, moderately differentiated and poorly differentiated).

The Kaplan-Meier method was used to estimate all survival distributions, and the log-rank test was used to test the differences between survival curves. Cox proportional hazard models were fitted to estimate hazard ratios to assess association of factors with each endpoint.

The definitions of the clinical endpoints used are as follows:

- Overall survival (OS) is the duration from index treatment (surgery) to death from any cause. Alive patients were censored at their date of last follow-up.

- Disease-free survival (DFS) is the duration from surgery to first relapse, irrespective of site. Dead patients without disease recurrence were censored at their dates of death. Alive patients without evidence of the relapse measured were censored at their date of last follow-up.

- Distant Metastases-free interval (DMFI) is the duration from surgery to first distant metastasis. Dead patients without disease recurrence were censored at their dates of death. Alive patients without evidence of the relapse measured were censored at their date of last follow-up.

- Locoregional Recurrence-free Interval (LRFI) is the duration from index treatment to first locoregional relapse. Dead patients without disease recurrence were censored at their dates of death. Alive patients without evidence of the relapse measured were censored at their date of last follow-up.

2.5 Ethics

The research plan was approved of by the institution review boards (IRB) of both the Singapore General Hospital and National Cancer Centre, Singapore.

CHAPTER 3

RESULTS

3.1 Clinical data

Fifty-one oral tongue squamous cell carcinoma patients were included in the study population. The median age was 57 years (range 18 – 89 years). There were more males (69%) than females. Twelve tumours (24%) were well differentiated, 35 (68%) moderately differentiated and 4 (8%) poorly differentiated. Twenty-five patients (49%) had nodal metastasis.

Among patients with non-missing data, 19 patients (49%) had perineural invasion and 8 (19%) had angioinvasion. Most patients had negative surgical margins (46 patients, 92%), and 20 (39%) patients had adjuvant therapy. Among the patients receiving adjuvant therapy, 14 (70%) were treated with radiotherapy and the remaining 6 (30%) had concurrent chemoradiation therapy. Patient and tumour characteristics are shown (Table 1).

Table 1. Demographic and histopathologic characteristics of all patients

Variable	No.	%
Total	51	100.0
Age at index treatment, years		
Median	57.0	
Range	18.8 – 89.7	
Gender		
Female	16	31.4
Male	35	68.6
T stage*		
T1	17	33.3
T2	24	47.1
T3	8	15.7
T4a	2	3.9
Nodal disease		
No	26	51.0
Yes	25	49.0
Overall stage*		
I	12	23.5
II	11	21.6
III	12	23.5
IVA	16	31.4
Angioinvasion		
No	35	81.4
Yes	8	18.6
Perineural invasion		
No	20	51.3
Yes	19	48.7
Tumour grade		
Well differentiated	12	24.0
Moderately differentiated	34	68.0
Poorly differentiated	4	8.0
Resection margins		
Negative	46	92.0
Positive	4	8.0
Adjuvant therapy		
No	31	60.8
Yes	20	39.2
Among patients with adjuvant therapy:	*20*	*100.0*
Radiotherapy	14	70.0
ChemoRT	6	30.0
Follow-up since index treatment, years		
Median	4.2	
Range	0.1 – 8.9	

*Based on AJCC version 6 pathological staging.

3.2 Expression of CD44s in oral tongue SCC

Microscopically, the oral tongue SCCs demonstrated proliferation of malignant epithelial cells arranged in solid sheets, nests, islands and cords invading the connective tissue with a variable degree of keratinization.

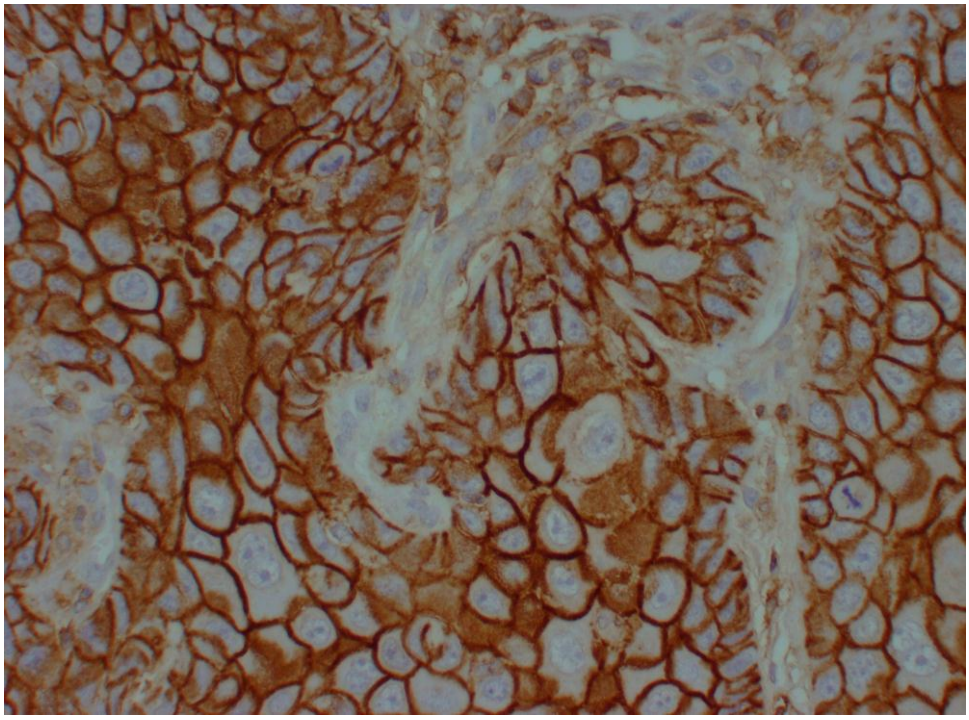
Immunoexpression of CD44s was noted in all oral tongue SCC specimens studied. The pattern of cellular distribution was membranous, and CD44s was expressed both by tumour epithelial cells and basal cells of keratinocytes.

CD44s expression was classified according to intensity of tumour cell staining, based on a semi-quantitative visual estimate to a 3-point scale (strong 3+; moderate 2+; weak 1+) (Figure 4), and percentage of tumour cells expressing CD44s. The median percentage of immunohistochemical staining of CD44s was 50% (range 5% to 100%). Strong staining intensity was demonstrated in 52.9%, moderate staining intensity in 41.2%, and 5.9% stained weakly for CD44s (Table 2).

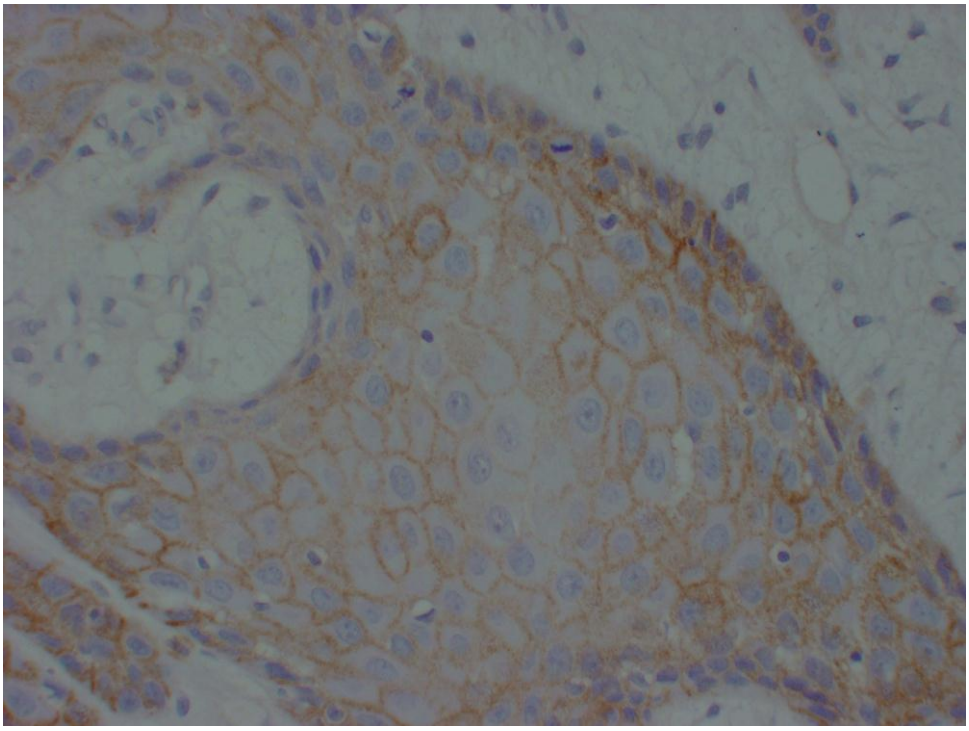
Table 2. IHC expression of CD44s in oral tongue SCC specimens

	No.	%
Total	51	100
Intensity		
Weak	3	5.9
Moderate	21	41.2
Strong	27	52.9
Percentage		
Median	50	
Range	5 – 100	

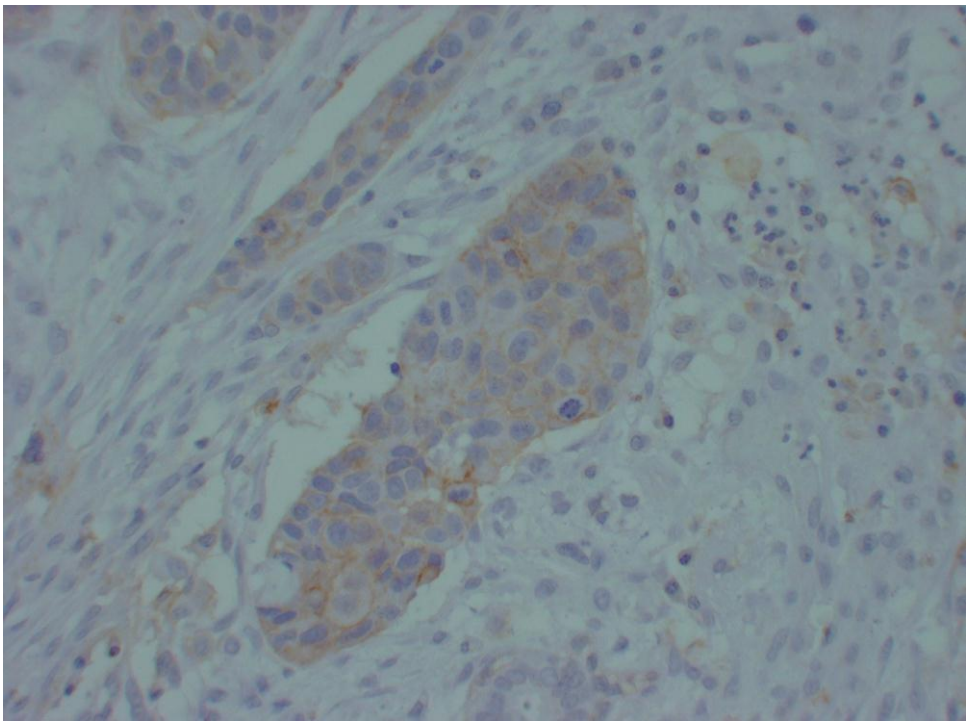
Figure 4. Immunohistochemical expression of CD44s in oral tongue SCC



a) Strong IHC staining intensity of CD44 s (3+ intensity)



b) Moderate IHC staining intensity of CD44s (2+ intensity)



c) Weak IHC staining intensity of CD44s (1+ intensity)

3.3 Correlation of CD44s expression with histopathologic features

The association of CD44s expression, both intensity of staining and percentage staining, with the following histopathological characteristics were examined (Table 3):

- Tumour grade (1: well differentiated, 2: moderately differentiated, 3: poorly differentiated)
- Presence of nodal disease
- Angioinvasion
- Perineural invasion

There was no significant association between CD44s staining percentage or intensity with histopathologic characteristics such as presence or absence of nodal metastasis, angioinvasion or perineural invasion.

Tumours that were moderately or poorly differentiated were more likely to show strong CD44s staining than well-differentiated tumours (60.5% vs 33.3 %, p = 0.08), although this did not reach statistical significance.

Table 3. Association of CD44s expression with histopathologic characteristics

	Tumour Grade						
	Total		Well differentiated		Moderately / Poorly differentiated		P-value
	No	%	No	%	No	%	
Total	50	100.0	12	100.0	38	100.0	
Intensity							
Weak	3	6.0	2	16.7	1	2.6	0.08
Moderate	20	40.0	6	50.0	14	36.8	
Strong	27	54.0	4	33.3	23	60.5	
Percentage							
Median	45		40		45		0.72
Range	5 – 100		10 – 100		5 – 90		-

	Tumour Grade								
	Total		Well differentiated		Moderately differentiated		Poorly differentiated		P-value
	No	%	No	%	No	%	No	%	
Total	50	100.0	12	100.0	34	100.0	4	100.0	
Intensity									
Weak	3	6.0	2	16.7	1	2.9	0	-	0.28
Moderate	20	40.0	6	50.0	13	38.2	1	25.0	
Strong	27	54.0	4	33.3	20	58.8	3	75.0	
Percentage									
Median	45		40		40		90		0.32
Range	5 – 100		10 – 100		5 – 90		10 – 90		-

	Presence of Nodal Disease						
	Total		No		Yes		P-value
	No	%	No	%	No	%	
Total	51	100.0	26	100.0	25	100.0	
Intensity							
Weak	3	5.9	3	11.5	0	-	0.32
Moderate	21	41.2	10	38.5	11	44.0	
Strong	27	53.0	13	50.0	14	56.0	
Percentage							
Median	50		40		50		0.30
Range	5 – 100		5 – 100		10 – 90		-

	Presence of Angioinvasion						
	Total		No		Yes		P-value
	No	%	No	%	No	%	
Total	43	100.0	35	100.0	8	100.0	
Intensity							
Weak	3	7.0	2	5.7	1	12.5	0.38
Moderate	17	39.5	13	37.1	4	50.0	
Strong	23	53.5	20	57.1	3	37.5	
Percentage							
Median	40		50		30		0.10
Range	5 – 100		5 – 100		10 – 80		-

	Presence of Perineural Invasion						
	Total		No		Yes		P-value
	No	%	No	%	No	%	
Total	39	100.0	20	100.0	19	100.0	
Intensity							
Weak	2	5.1	2	10.0	0	-	0.21
Moderate	16	41.0	6	30.0	10	52.6	
Strong	21	53.8	12	60.0	9	47.4	
Percentage							
Median	50		50		40		0.76
Range	5 – 100		5 – 100		10 – 90		-

3.4 Correlation of CD44s expression with clinical outcomes

Survival data was analysed for all 51 patients included in the study population.

The median follow-up time was 4.2 years (range 0.1 – 8.9 years). Univariate Cox regression analysis was performed to assess the strength of association of each clinicopathological parameter and CD44s expression (percentage staining and intensity) with overall and disease-free survival, as well as distant recurrence-free and locoregional recurrence-free intervals.

3.4.1 Overall Survival

Strong intensity of immunohistochemical staining for CD44s and the presence of nodal metastases were identified as possible prognostic factors on univariate analysis (Table 4). On multivariate analysis, strong staining was found to be independently associated with improved overall survival and the presence of nodal metastases was an independent poor prognostic factor after adjustment for the relevant covariates (Table 5).

Kaplan-Meier survival curves indicate that strong CD44s expression had significantly better overall survial compared to those with moderate/weak staining (log rank P = 0.04, Figure 5).

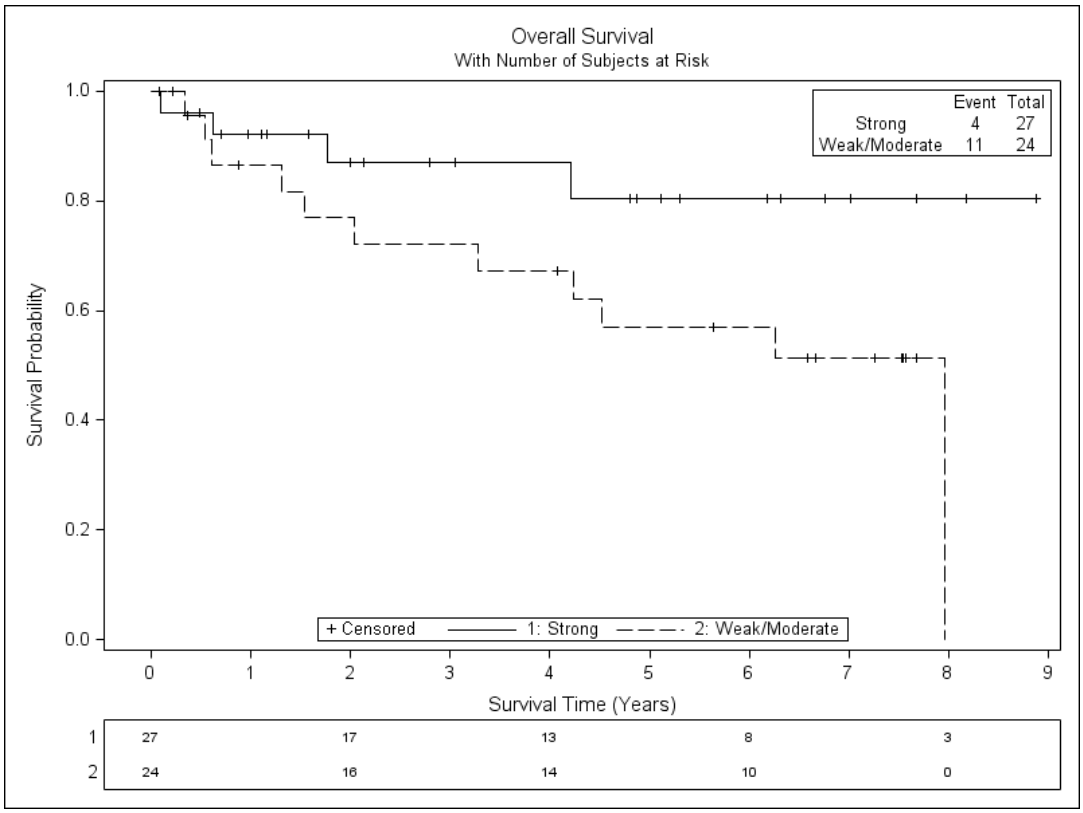
Table 4. Univariate Cox regression analysis for overall survival

Variable	Overall survival		
	HR	95% CI	p
CD44s intensity (strong)	0.32	0.10-1.03	0.06
CD44s percentage	0.99	0.97-1.01	0.20
Gender (male)	0.53	0.19-1.50	0.23
T stage (T3/4a vs T1/2)	2.22	0.76-6.53	0.15
Nodal mets (present)	2.81	0.94-8.35	0.07
Stage (III/IVa vs I/II)	2.11	0.71-6.27	0.18
Angioinvasion	1.42	0.44-4.57	0.55
Perineural invasion	1.79	0.60-5.37	0.30
Resection margins (involved)	2.43	0.67-8.78	0.18
Adjuvant therapy	0.99	0.35-2.78	0.98

Table 5. Multivariate Cox regression analysis of overall survival

Variable	Categories	No of events / patients	HR (95% CI)	P-value^
CD44 intensity	Weak / Moderate Strong	11 / 24 4 / 27	1 0.27 (0.09 – 0.87)	0.028
Nodal disease	No Yes	5 / 26 10 / 25	1 3.45 (1.13 – 10.53)	0.030

Figure 5. Kaplan-Meier estimate of overall survival in oral tongue SCC based on CD44s staining intensity – strong vs moderate/weak staining. Log rank *P* = 0.04



3.4.2 Disease-free interval

Univariate analysis was performed to assess the strength of association of each clinicopathological paramter and CD44s expression with disease-free interval. Angioinvasion and the presence of nodal metastases were identified as likely significant independent prognostic factors (Table 6). On multivariate analysis, the presence of angioinvasion showed to be an independent poor prognositic factor for disease-free interval, while the presence of nodal metastases was not significantly associated with disease-free interval (Table 7).

Table 6. Univariate Cox regression analysis for disease-free interval

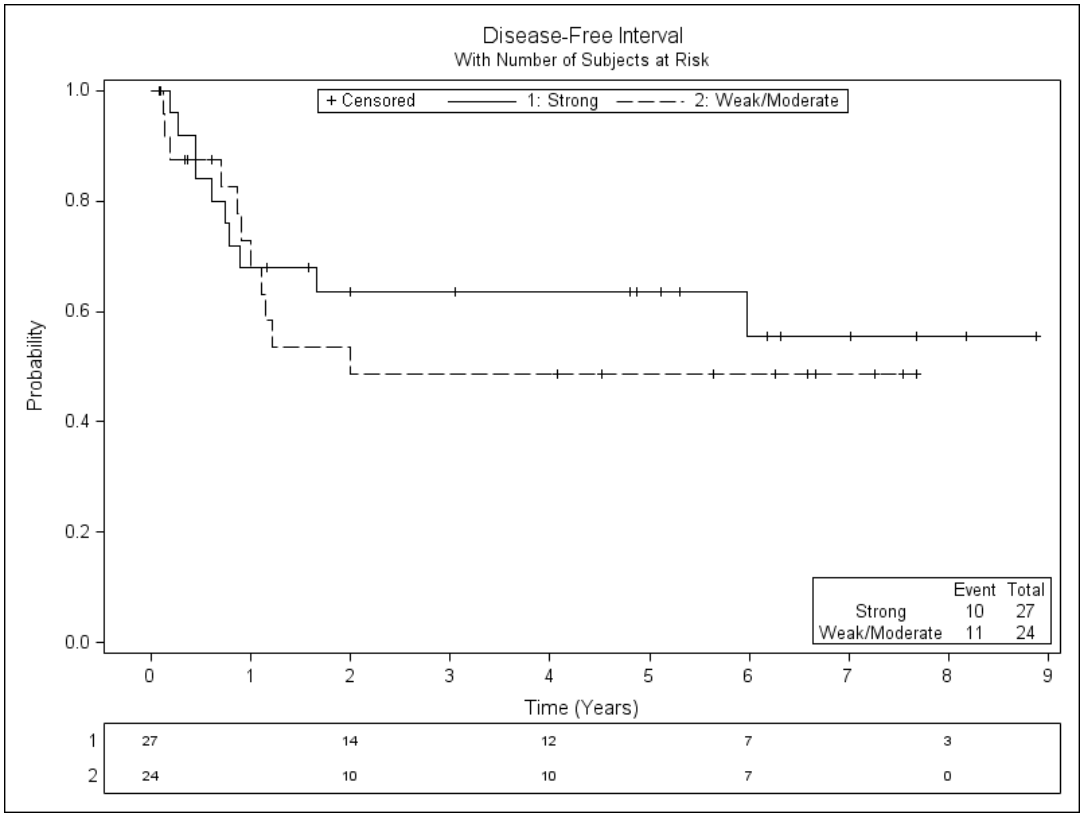
Variable	Disease-free survival		
	HR	95% CI	p
CD44s intensity (strong)	0.79	0.33-1.85	0.58
CD44s percentage	1	0.98-1.01	0.52
Gender (male)	0.49	0.20-1.19	0.12
T stage (T3/4a vs T1/2)	1.10	0.37-3.28	0.87
Nodal mets (present)	2.29	0.94-5.55	0.067
Stage (III/IVa vs I/II)	1.65	0.68-4.00	0.27
Angioinvasion	3.77	1.46-9.70	0.006
Perineural invasion	1.70	0.67-4.34	0.26
Resection margins (involved)	1.24	0.29-5.38	0.77
Adjuvant therapy	0.84	0.35-2.02	0.69

Table 7. Multivariate Cox regression analysis for disease-free interval

Variable	Categories	No of events / patients	HR (95% CI)	P-value^
Nodal disease	No	8 / 21	1	0.635
	Yes	12 / 22	1.27 (0.48 – 3.34)	
Angioinvasion	No	13 / 35	1	0.018
	Yes	7 / 8	3.43 (1.24 – 9.47)	

The immunohistochemical expression of CD44s is not associated disease-free interval. Figure 6 shows the Kaplan Meier estimate of strong staining intensity of CD44s vs moderate/weak staining intensity (Log-rank P = 0.58).

Figure 6. Kaplan-Meier estimate of disease-free interval in oral tongue SCC based on CD44s staining intensity – strong vs moderate/weak staining. Log rank *P* = 0.58



3.4.3 Distant metastasis-free interval

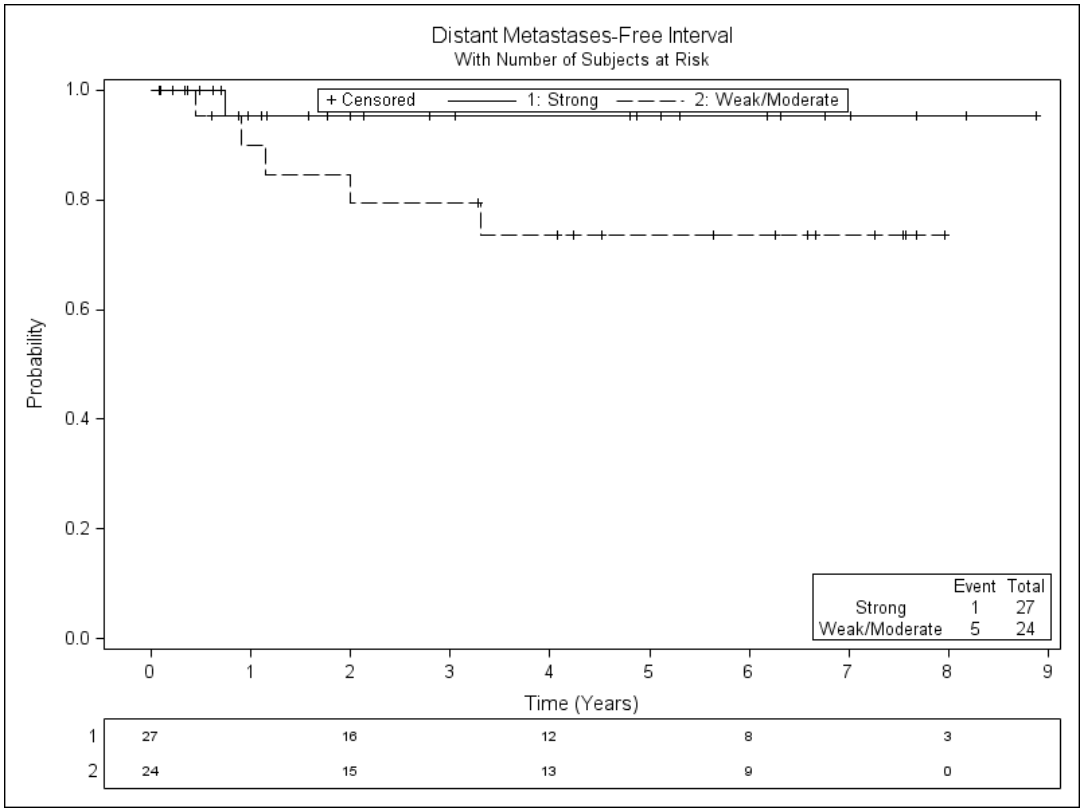
The risk of distant metastasis was lowered by 4% with every unit increase in CD44s percentage staining (HR 0.96, 95% CI 0.92 – 1.00; P =0.08) (Table 8). The other variables analysed were not associated with distant metastasis-free interval.

Table 8. Univariate Cox regression analysis of distant metastasis-free interval

Variable	Distant metastasis-free interval		
	HR	95% CI	P
CD44s intensity (strong)	0.19	0.02-1.59	0.12
CD44s percentage	0.96	0.92-1.00	0.08
Gender (male)	0.75	0.14-4.09	0.74
T stage (T3/4a vs T1/2)	2.37	0.43-12.9	0.32
Nodal mets (present)	2.87	0.52-15.7	0.23
Stage (III/IVa vs I/II)	2.13	0.39-11.7	0.38
Angioinvasion	0.84	0.10-7.22	0.88
Perineural invasion	1.42	0.28-7.11	0.67
Resection margins (involved)	2.09	0.24-17.9	0.50
Adjuvant therapy	1.46	0.29-7.25	0.64

Kaplan-Meier estimates of distant-metastasis free interval showed no difference between strong staining intensity and moderate/weak staining (Log-rank P = 0.09) (Figure 7).

Figure 7. Kaplan-Meier estimate of distant metastasis-free interval in oral tongue SCC based on CD44s staining intensity – strong vs moderate/weak staining. Log rank *P* = 0.09)



3.4.4 Locoregional recurrence-free interval

On univariate analysis, strong CD44s expression correlated with improved locoregional recurrence-free interval (HR 0.25 95%CI 0.07-0.93, p=0.53). The presence of angioinvasion is also a poor prognostic factor for LRFI (HR 3.79 95%CI 1.34 – 10.72, p=0.012) (Table 8). On multivariate analysis, both remained independently associated with locoregional recurrence-free interval after adjustment for covariates (Table 9).

Table 9. Univariate Cox regression analysis for locoregional recurrence-free interval

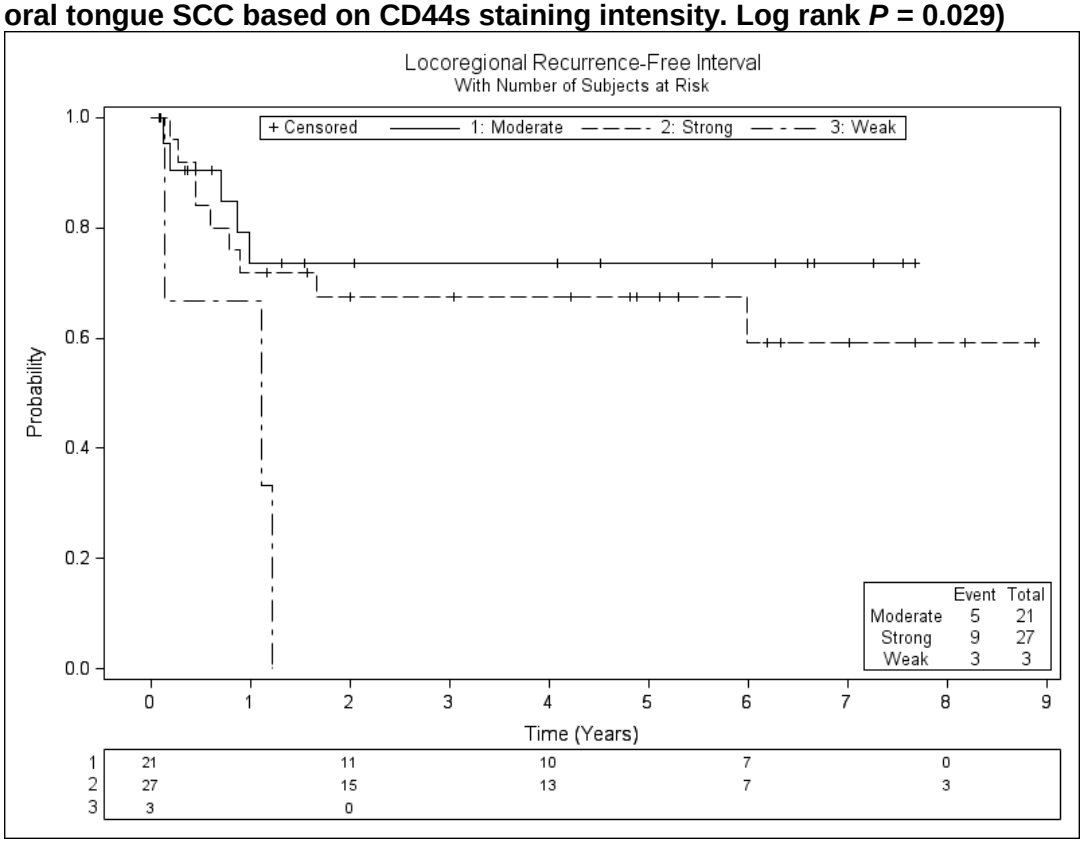
Variable	Locoregional recurrence-free interval		
	HR	95% CI	p
CD44s intensity (strong)	0.25	0.07-0.93	0.053
CD44s percentage	1.0	0.98-1.02	0.962
Gender (male)	0.44	0.17-1.16	0.095
T stage (T3/4a vs T1/2)	0.56	0.13-2.46	0.443
Nodal mets (present)	1.47	0.57-3.84	0.428
Stage (III/IVa vs I/II)	1.08	0.42-2.82	0.871
Angioinvasion	3.79	1.34-10.7	0.012
Perineural invasion	1.38	0.48-3.97	0.546
Resection margins (involved)	0.68	0.09-5.17	0.711
Adjuvant therapy	0.75	0.28-2.03	0.569

Table 10. Multivariable Cox regression analysis of locoregional recurrence-free interval

Variable	Categories	No of events / patients	HR (95% CI)	P-value^
CD44 intensity	Weak	3 / 3	1	0.029
	Moderate	5 / 17	0.12 (0.03 – 0.59)	
	Strong	8 / 23	0.22 (0.05 – 0.87)	
Angioinvasion	No	10 / 35	1	0.004
	Yes	6 / 8	5.40 (1.74 – 16.76)	

The Kaplan-Meier survival plot of locoregional recurrence-free interval by CD44s staining intensity is shown in figure 8. There is a statistical significance in LRFI between patients with different CD44s staining intensity. (Log-rank P = 0.029)

Figure 8. Kaplan-Meier estimate of locoregional recurrence-free interval in oral tongue SCC based on CD44s staining intensity. Log rank *P* = 0.029)



CHAPTER 4

DISCUSSION

4.1 Association of CD44s expression with histopathological features

Since CD44 has been associated with head and neck cancers, its function has been of great interest to researchers. Its purported roles have ranged from being a cell-surface docking receptor having a role in cell-cell adhesion, to being an initiator of metastasis and even as a cell surface marker for head and neck cancer cells with stem-like qualities. Its exact function has yet to be defined, and researchers have studied this aspect extensively. As a starting point in elucidating the role of CD44, investigators have often explored the expression of CD44 with histopathological features. In the treatment of head and neck cancers, several histopathological features are known to be associated with a poorer outcome. These adverse features are commonly used as surrogates to indicate an aggressive biology, and adjuvant therapy is accordingly tailored to include chemotherapy with radiotherapy for better disease control. They include tumour grade, nodal metastases, extracapsular nodal spread, angioinvasion and perineural invasion.

A systematic review of the available studies investigating CD44 expression in head and neck cancers located 8 studies that sought to determine if CD44 expression correlated with any histopathologic features. These studies varied in the tumour subsites being investigated, CD44 isoform being identified, as well as the method of identification and scoring parameters. They were similar in that CD44 (or its isoforms) expression

was quantified, and statistical methods were used to find associations with various histological or staging parameters.

We investigated if CD44s expression by immunohistochemistry, either intensity of staining or percentage staining, was associated with the aforementioned adverse histopathological features and found no significant association with any of the adverse histological features. There was a trend towards an association with tumours that were moderately or poorly differentiated being more likely to show strong CD44s staining than well-differentiated tumours (61% vs 33 %, p = 0.083), but this did not reach statistical significance.

Similar to our study, two studies Kawano et al and Gonzales-Moles et al found no association between CD44 expression and histopathological features.[67, 69] Kawano et al attempted to study both clinical outcome and histologic association of CD44s and CD44v6 in 57 oropharyngeal carcinomas. Most of their patients underwent surgery and radiotherapy, and the follow up ranged from 5 to 129 months. Kawano and coworkers found that CD44s and CD44v6 mainly stained the basal lamina and fibrous connective tissue, and semi-quantitative scoring (<5%; 6-25%; 26-50% and >50%) separated the tumours into 4 groups. No significance between staining and tumour grade or stage were found.[69] In the study by Gonzales-Moles et al, 36 tongue SCCs were assessed. Standard CD44 was stained for, and only cells with membrane staining were counted. Expression was categorized as low (<50% of tumour cells) or high (>50%

of tumour cells). The authors found no association between CD44s expression and histopathology.[67]

Several researchers have reported that low expression, or down-regulation of CD44 is associated with advanced tumour grade and nodal metastasis.[63-66] These outcomes have largely been attributed to the role of CD44 as a cell-cell adhesion molecule, where its down-regulation may result in cell detachment with consequent metastasis to the lymph nodes.[63-66]

In brief, Kosunen et al. categorised CD44 expression by the intensity (1+ to 4+) and distribution of staining (homogenous vs irregular). 138 samples of oral squamous cell cancer were analysed. Irregular staining of CD44 was associated with poor tumour differentiation and a worse clinical stage.[63] Sato et al. studied specifically the expression of CD44v9 in 120 specimens of SCC tongue. CD44v9 expression was grouped according to staining patterns in nests of tumour cells. Three groups were defined: all cells in the periphery of nests were distinctly positive, a reduction in expression in the periphery of tumour nests, and completely negative expression in one or more nests. This group found that decreased expression of CD44v9 was associated with increased lymph node metastasis. They postulated that the CD44v9 molecule may play an important role in cell-cell adhesion, and its downregulation may result in cell detachment from tumour nests and subsequent metastasis.[64] Stoll et al. studied the expression of 5 CD44 isoforms (v4, v5, v6, v7, v9) in 99

primary, non-metastatic, oral and oropharyngeal SCC. Tumours were evaluated at 100-fold magnification. Cells were counted at each high-powered field and a tumour was deemed to have a down regulation of CD44 if expression of CD44 was less than 50%. A decreased expression of CD44v9 was found to correlate with higher histological grade.[65] Rodrigo and co-workers looked at CD44s and CD44v6 in 101 laryngeal tumours. CD44 expression was based on the percentage of cells with membranous staining (0-100%). Both CD44s and CD44v6 downregulation were associated with poorer differentiation in the tumours.[66]

Other groups have reported directly conflicting data, where up-regulation, or strong CD44 expression is associated with the malignant phenotype. Joshua et al. recently evaluated the association of histopathologic features with CD44 expression using FACS to determine the percentage of tumour expressing the cell surface antigen, CD44.[72] Twenty-two squamous cell carcinomas from various sites of the head and neck were examined. They noted a trend towards an inverse correlation between tumour grade and mean $CD44^+$ frequency, that is, tumours with a greater percentage of cells expressing CD44 tended to be of poorer differentiation.[72] This finding was similar to that noted in our series of 51 oral tongue SCCs. This group also noted that a higher frequency of $CD44^+$ cells was found in patients with perineural and angiolymphatic invasion, but these trends were not found to be statistically significant.[72]

Mori and coworkers wanted to establish the role CD44 as a biological marker for metastasis. The association between CD44 expression and lymph node metastasis was compared in 86 specimens of tongue SCC.[68] Among the 54 cases that had strong staining, 44.4% had metastatic disease. Compared with 23.3% with metastatic disease in weak staining and no metastasis in tumours with no CD44 staining, a statistically significant association between high CD44 expression and increased lymph node metastasis was found. Although this study did not directly explore clinical outcome, lymph node metastasis has been established as an independent prognostic factor for survival in HNSCC. Recent research has shed light on the functional effects of CD44 as a adhesion/homing molecule involved in pro-oncogenic signaling pathways and being linked to the metastatic potential of many cancers.[73] The process of cancer metastasis is best thought of as an orchestrated series of steps that depend first on the growth and invasive potential of the primary tumour. In a cancer, cells first lose adhesiveness and detach from the primary site. These detached, viable cells – metastatic cells, then penetrate the lymphatic and vascular systems by transendothelial migration and circulate until adhesion molecules and chemoattractants induce extravasation and cell accumulation in a distant organ to establish a metastatic deposit. While the binding capacity of CD44 has been invoked to support a decreased detachment of tumour cells, and hence decreased metastatic potential, the *functional* effects of CD44 binding to its various ligands- hyaluronan, collagen, fibronectin, growth factors etc, have been linked to an increased migratory capacity and vascular invasiveness.[74] In

further support of its role in cancer dissemination, CD44 has also been associated with epithelial-mesenchymal transition, and the inhibition of CD44 with specific antibodies reduces EMT and metastasis formation.[73] It also has a role in directing homing of circulating tumour cells to permissive metatstatic sites, and is currently being investigated as a therapeutic target to prevent metastasis.[75]

A summary of the studies that investigated CD44 expression and its association with histopathological features is presented in Table 11.

Table 11. Studies that examined the association of CD44 expression in head and neck SCC with histopathological features

Study	Site	No. of Patients	CD44 Isoform(s)	Method of analysis	Scoring method	CD44 Characteristics	Histopathologic Correlation
Kawano 2004 [84]	Oropharynx	57	CD44s CD44v6	IHC	% Tumour staining for CD44	High CD44s, CD44v6 expression	No histopathologic association
Gonzalez 2007 [82]	Tongue	36	CD44s	IHC	% Tumour staining for CD44	Low CD44 expression (<50%)	No histopathologic associations
Joshua 2012 [80]	HNSCC	31	Pan CD44	FACS	% Tumour cells expressing CD44	Higher % CD44 expression	Poorer tumour differentiation Trend towards perineural invasion
Mori 1998 [83]	Oral Cavity	86	CD44s	IHC	Intensity of CD44 staining	Strong staining intensity for CD44	Increased LN metastasis
Sato 2000 [79]	Tongue	120	CD44v9	IHC	Pattern of staining – All/irregular/none	Low CD44v9 expression	Increased LN metastatsis
Stoll 1999 [80]	Oral Cavity Oropharynx	99	CD44v4 CD44v5 CD44v6 CD44v7 CD44v9	IHC	% Tumour stainging for CD44	Low CD44v9 expression	Poor tumour differentiation
Rodrigo 2002 [81]	Larynx	101	CD44s CD44v6	IHC	% Tumour with membrane staining for CD44	Low CD44s and CD44v6 expression	Poor tumour differentiation
Kosunen 2007 [78]	Oral	138	CD44s	IHC	Intensity & regularity of staining	Low expression and irregular staining	Poor tumour differentiation Advanced T stage

4.2 CD44s as a prognostic marker

The prognostication of oral tongue SCC is to date based upon clinicopathological grading systems such as the TNM staging of the AJCC classification. However, survival varies significantly even within these groups, and new biological markers to more accurately predict outcomes are constantly explored. CD44, a putative HNSCC cancer stem cell marker, a molecule implicated with tumour progression and metastasis, and with an apparent key role in cell-cell / cell-matrix adhesion make it an interesting candidate for study as a prognostic marker.

We looked at four clinical outcomes of importance – overall survival, disease-free interval, distant disease-free interval and locoregional disease-free interval. In the management of head and neck cancers, perhaps more than in cancers of other organs, the ability to afford good locoregional control is paramount as the functional deficits with tumour recurrence often directly affect speech and swallowing with resultant significant morbidity and mortality.

Regression analyses were performed to determine if gender, tumour size, nodal status, disease stage, angioinvasion, perineural involvement, adjuvant therapy or CD44s expression (intensity and percentage staining) were associated with the four survival outcomes of interest.

The immunohistochemical expression of CD44s in oral tongue SCC was found to have prognostic significance. Staining intensity of CD44s was shown to be a better prognostic marker than the percentage of CD44s positive cells. Strong CD44s staining was associated with better overall survival when compared with weak/moderate staining intensity. Strong CD44s staining was also associated with better locoregional recurrence-free interval and remained an independent positive prognostic factor on multivariate analysis.

Our findings, that strong staining intensity of CD44s is an independent positive prognostic marker for overall and locoregional disease-free survival, are in conflict with our expectations that as a cancer stem cell marker, a greater expression of CD44 would be a poor prognostic marker. According to the cancer stem cell theory, a higher stem cell burden, as reflected by greater CD44s expression, should correlate with a worse outcome in terms of recurrence and survival. However, this is clearly not the case and it is likely that high CD44s expression detected through our technique is a reflection of an alternative function of CD44 in the cell.

A review of published studies that examined CD44 expression with survival outcomes yielded, again, conflicting results. Studies that reported similar findings to the current series showed weak staining, irregular staining or low expression of CD44 to be a poor prognostic marker for disease recurrence or overall survival.[63-67] In contrast, a recent study by Lindquist et al examined 102 oropharyngeal (tonsil and base of tongue)

SCC to determine CD44s expression using IHC.[76] Similar to our study, both the intensity of staining and percentage of positively stained cells were evaluated by a senior pathologist and semi-quantitatively into 4 groups each (Intensity of staining: 0 to 3+ equal to absent, mild, moderate and strong; Percentage of positive cells scaled to 0, 1-10%, 11-49%, and >50%). They found on multivariate analysis that a high intensity of CD44 staining was a strong, independent indicator of a poor prognosis.[76]

A tabulated summary of the studies that investigated CD44 expression with clinical outcomes is presented in Table 12.

One of the dilemmas in reviewing the literature on CD44 as a potential biomarker for HNSCC is the variability of the parameters investigated. The 3 main variables are: firstly, the CD44 isoform being investigated; secondly, the method of determining CD44 expression; thirdly, the tumour subsite within the head and neck.

CD44 is a molecule with great structural diversity. It has multiple isoforms and undergoes posttranslational modifications with resultant multiplicity of function. Furthermore, it may require specific stimulation for activation. In vitro, there is evidence that it exists in the active, inducible and inactive states. Active CD44 constitutively binds hyaluronan, while inducible CD44 bind hyaluronan weakly or not at all, unless stimulated; and inactive CD44 does not bind hyaluronan even in the presence of inducing factors.[77] All these permutations, and the high likelihood that each CD44 isoform has its

independent function, contribute to the complexities in clearly elucidating the role of CD44 because meaningful comparisons between different series that use differing isoforms is not possible.

In this work, the association between the IHC expression of the standard isoform of CD44, CD44s, and clinical outcomes was explored. It might well be that CD44s is not the splice variant that most appropriately reflects CD44 role as a stem cell marker and the actual function of CD44s in oral tongue SCC in our experiments is still unknown.

The use of IHC to quantify CD44s expression may also not be most ideal method to explore its role as a cancer stem cell marker. In research demonstrating CD44 as a cell surface marker for cancer stem cells, flow cytometry analysis is often used to quantify the proportion of tumour epithelial cells expressing the molecule. FACS is quantitative for the given volume of tumour, and excludes stromal and inflammatory cells that may express CD44. Furthermore, a pan-CD44 antibody, instead of quantifying a specific variant is used. In contrast, IHC is a semi-quantitative evaluation based on an individual observer's estimate of CD44 expression on a histological section that may have limited ability to represent the entire tumour. It is also difficult on IHC staining to quantitatively differentiate between staining of tumour and non-tumour cells. Yet, despite recognizing the limitations of IHC, the reason why we, and many other studies, explored the use of IHC to quantify CD44 expression is because it is the method of quantifying most likely to be of clinical relevance. However,

differences in the methods of categorizing the immunoexpression of CD44 also exist and add further to the difficulty of evaluating the existing literature. Determined arbitrarily by individual investigators, some looked at the regularity of staining patterns, others the percentage of cells that stain, and yet others that quantify the staining intensity – all with differing categories. Such differences effectively preclude meaningful comparisons between published studies. Perhaps studies to examine if FACS and IHC give comparable findings should be done, as should the best way to quantify immunoexpression be identified.

Certainly the standardization of techniques and approaches to analyse the heterogenous CD44 molecule on human cancers across laboratories worldwide may help obtain more consistent data from patient specimens. With regards to head and neck cancers, evaluation based on individual subsites is also likely to distil further, relevant results. Too often, entire cohorts of head and neck cancers without a subsite focus are analysed, but as most clinicians are aware, tumour behavior exhibits significant subsite specificity.

Table 12. Studies that examined the association of CD44 expression in head and neck SCC with clinical outcomes.

Study	Site	No. of Patients	CD44 Isoform(s)	Method of analysis	Scoring method	CD44 Characteristics	Association with Recurrence	Association with overall survival
Gonzalez 2007 [82]	Tongue	36	CD44s	IHC	% Tumour staining for CD44	Low CD44 expression (<50%)	No Data	Decreased survival
Sato 2000 [79]	Tongue	120	CD44v9	IHC	Pattern of staining –All/irregular/none	Low CD44v9 expression	No Data	Decreased survival
Stoll 1999 [80]	Oral Cavity Oropharynx	99	CD44v4 CD44v5 CD44v6 CD44v7 CD44v9	IHC	% Tumour stainging for CD44	Low CD44v9 expression	Earlier recurrence	Decreased survival
Rodrigo 2002 [81]	Larynx	101	CD44s CD44v6	IHC	% Tumour with membrane staining for CD44	Low CD44s and CD44v6 expression	Higher recurrence rate with CD44s	Not associated
Kosunen 2007 [78]	Oral	138	CD44s	IHC	Intensity & regularity of staining	Low expression and irregular staining	Earlier recurrence	Decreased survival
Lindquist 2012 [84]	Oropharynx	102	CD44s	IHC	% Cells staining for CD44s and Intensity of CD44s expression	Stronger CD44s staining intensity	No Data	Decreased Survival
Kawano 2004 [84]	Oropharynx	57	CD44s CD44v6	IHC	% Tumour staining for CD44	High CD44s, CD44v6 expression	No Data	Decreased survival
Joshua 2012 [90]	HNSCC	31	Pan CD44	FACS	% Tumour cells expressing CD44	Higher % CD44 expression	Higher recurrence rate	Decreased survival

4.3 Directions for future research

As basic science research delves further to clarify the function of the CD44 protein in head and neck cancers, there are perhaps 3 main applications in the arena of clinical investigation in which CD44 and its isoforms may prove useful. The first, as we explored with CD44s in oral tongue cancer, is its role as a prognostic marker. Refinement of the choice of technique employed to measure expression and identifying the most indicative splice variant has potential to greatly aid prognosis.

The second potential area of clinical application is as a marker of radioresistance and/or chemoresistance and hence a predictor to treatment failures. The underlying concept to this is the cancer stem cell hypothesis. Cancer stem cells of breast cancer cell lines and glioblastoma stem cells in vivo have been shown to be radioresistant. In glioblastoma, these cells accumulated after radiation therapy, and by examining known molecular markers of radiation damage, it was concluded that the group of cancer initiating cells defined by $CD133^+$ expression could repair DNA damage more efficiently and hence survive. Perhaps more interesting was the ability to radiosensitize $CD133^+$ cells by inhibiting DNA damage check point kinases Chk1 and Chk2. Should CD44 as a cancer stem cell marker in head and neck cancer have similar ability, the potential clinical implications are significant. Potentially we may identify the patients at risk of failure with radiotherapy and pre-emptively select a different treatment

modality or, should we be able to target and effect radiosensitization, do so prior to treatment. A similar trend of clinical application exists for head and neck cancer with chemotherapy where CD44 – hyaluronan signaling has been shown to play a role in cisplatin resistance. We had hoped to investigate this aspect by determining if CD44s expression had a correlation with success of treatment with upfront concurrent chemoradiotherapy. Our initial dataset of patients with locally advanced head and neck cancers treated primary chemoradiotherapy might have shed light on a possible association with treatment failure. Unfortunately we failed to optimize a suitable IHC protocol for the archived specimens from 1996 – 2002 and we were compelled to change our experimental plan and forsake this aspect.

The third clinical area of great potential is the use of CD44 as a target for therapy. As a cell surface antigen, its expression is perhaps not sufficiently specific to allow for precise targeting. This is due to its ubiquitous expression on a large variety of cells. This is illustrated in the phase I study reported by Riechelmann et al., that investigated the use of the antimitotic agent, mertansine, conjugated with the monoclonal antibody, bivatsuzumab, specific for CD44v6. The potent derivative, bivatuzumab mertansine, was used as a selective cytotoxic agent to target CD44v6 expressed by HNSCC in patients with recurrent or metastatic disease. Although effective, the side effects were significant due largely to the fact that CD44v6 is also expressed on dermal keratinocytes, and the trial reported one death from toxic epidermal necrolyis. Its use as a

therapeutic agent has since been curtailed. Nonetheless, this highlights the potential of CD44, and perhaps its more cancer-specific associated signaling pathways, as potential therapeutic targets for novel therapies.

4.4 CONCLUSION

This study examined the clinical implications of CD44s expression in a population of oral tongue SCC. It was based on the hypothesis that CD44, a putative cancer stem cell marker of head and neck SCC, may have prognostic significance in the clinical outcomes of patients with oral tongue SCC, be a potential biomarker for increased resistance to chemotherapy and radiotherapy, and may be associated with adverse histopathological features. We found instead, that CD44s is abundantly expressed in oral tongue SCC, and when strongly expressed, is a good prognostic marker for both overall survival and locoregional recurrence-free interval. Immunoexpression of CD44s is therefore unlikely to itself reflect the proportion of cancer stem cells within oral tongue SCC.

More coordinated research is needed to definitively define the role of CD44 and its variants in head and neck cancer.

CHAPTER 5

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